Aquaculture 307 (2010) 111–122

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Expression profile for metabolic and growth-related genes in domesticated and transgenic coho salmon (*Oncorhynchus kisutch*) modified for increased growth hormone production

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ARTICLE INFO

Article history:
Received 24 September 2009
Received in revised form 8 June 2010
Accepted 9 June 2010

Keywords:
Metabolism
Growth
Gene expression
Growth hormone
Transgenic
Coho salmon

ABSTRACT

To gain a better understanding of the aspects underlying growth in salmonids, quantitative expression analysis was performed for a number of genes related to muscle growth, metabolism, immunology and energy regulation in the liver and muscle of wild-type coho salmon (*Oncorhynchus kisutch*), domesticated coho salmon selected for growth, growth hormone-transgenic coho salmon fed to satiation, and growth hormone-transgenic coho salmon reared on restricted rations. In comparisons among these four experimental groups, our findings show a significant and correlative up-regulation in the expression of a number of muscle determination and development factors in full-fed (FF) transgenic and domestic coho salmon. Expression of several metabolic genes involved with amino acid turnover and utilization also was correlated between these two faster-growing groups. However, distinct differences between domesticated and FF-transgenic fish were found for some genes involved with muscle cell differentiation, innate immune system, metabolism, and amino acid regulation. Wild-type and ration-restricted growth hormone-transgenic fish correlated very highly in the expression of most genes tested, suggesting that limiting available energy reduces the effects of elevated growth hormone and down-regulates muscle and metabolic regulatory pathways similarly to the reduced potential for growth hormone production seen in wild-type fish.

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1. Introduction

The Food and Agriculture Organization of the United Nations (FAO) predicts that the growth of aquaculture will continue to rise and surpass capture fisheries in the near future (FAO, 2009). Recently, it was reported that aquaculture now provides more than half of the fish consumed globally (Naylor et al., 2009). In fact, the FAO (2009) suggests that within the next 20 years, aquaculture production will need to increase by at least 40 million tons/year in order to meet demand. Increasing concerns regarding the effects of aquaculture on the environment and use of unsustainable practices for rearing fish are forcing the industry to optimize production. For commercially valuable species, selective breeding programs have been initiated to develop faster, more efficiently growing strains of fish. Another method for improving growth in fish has been the development of transgenic strains designed to enhance weight gain and/or growth rate. Studies have shown that the genetic transformation of wild-type fish with growth hormone (GH) gene constructs can result in significant improvements in weight gain and growth rate (Du et al., 1992; Devlin et al., 1994; Nam et al., 2001; Rahman et al., 1998). However, domestication of fish (selected for fast growth) seems to interact with transgenic manipulation. In rainbow trout, expression of GH genes or treatment with exogenous GH protein had a reduced effect in a highly domesticated strain vs. wild-type, whereas a synergistic effect was seen for coho salmon (Devlin et al., 2001). Research has been initiated to characterize the expression at coincidental pathways involved with growth regulation between these two genetic systems.

In order to accurately determine the similarities and differences between selected domesticated fish, wild-type, and transgenic fish modified with enhanced growth hormone production, samples were taken from the liver and muscle of these groups and analyzed for changes in expression of specific genes. A preliminary study involved the use of microarrays to study expression differences in liver and muscle, and found that domesticated fish and growth hormone-transgenic strains modulate the expression of certain genes in a similar and strongly correlative manner (Devlin et al., 2009a). To further investigate the similarities and differences between these groups, and to gain a better understanding of energy partitioning, muscle growth, and metabolism, the expressions of several specific genes involved in development, regulation, metabolism, and degradation in muscle were

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0044-8486/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.aquaculture.2010.06.010

analyzed. In addition, genes involved in immunological, growth, metabolism and cellular protection functions in the liver have been examined.

In muscle, genes involved with differentiation and propagation were studied, including the conserved edge expressed protein (*cee*) and *Pax7*. *Cee* originally was identified in a genome-wide search for genes associated with myotube formation using pufferfish (Macqueen et al., 2007). In salmonids, *cee* has been found in the superficial layers of developing tissues and is believed to play a role in normal growth and development (Femandes et al., 2008). *Pax7* is expressed in quiescent satellite cells. When satellite cells are activated, *Pax7* is found co-expressed with *MyoD*. It is believed that *Pax7* is involved in maintaining muscle cell proliferation and preventing precocious differentiation (Zammit et al., 2006). In fish, it has been shown that dietary components and available energy can regulate muscle myogenesis through *Pax7* and other myogenic factors (Chapalamadugu et al., 2009).

Differences in muscle development have been examined using the muscle-specific genes *myogenin*, *MyoD*2, *Myf5*, *Mef2A*, and *Mef2C*. *Myogenin*, *MyoD* and *Myf5* are muscle regulatory factors (MRFs) that are members of a family of basic helix–loop–helix (bHLH) transcription factors known to regulate the transcription of other muscle genes (Roy et al., 2002). *MyoD* and *Myf5* are known to function in a partially redundant manner during the specification and proliferation of myoblasts (Braun et al., 1992; Rudnicki et al., 1993). *Mef2A* and *Mef2C* belong to a separate group of transcription factors (myocyte enhancer factor 2 family) and function together with MRFs to regulate muscle development and growth. It is believed that MEF2 proteins are mainly involved in regulating muscle differentiation, but not specification. The MEF2 proteins alone are not sufficient to induce myogenesis, but interact with MRF family proteins by forming heteroprotein complexes that control the transcription of numerous muscle structural genes, including myosin heavy and light chains, desmin, and troponins, as well as those encoding the bHLH MRFs themselves (Black and Olson, 1998). Myostatin is a secreted protein of the transforming growth factor β (TGFβ) family that in mammals has been demonstrated to act as an antagonist to muscle growth (Sharma, et al., 1999; Thomas et al., 2000). Several myostatin sequences have been characterized in salmonids with different expression patterns (Rescan et al., 2001; Roberts and Goetz, 2001). Two of these genes, *Tmyostatin1* and *Tmyostatin2*, were assessed on muscle isolated from fish samples in this study.

Recent research has started to more intensively analyze connections between factors involved with growth or metabolism (Stelling et al., 2002; Picah et al., 2009). In balancing energy partitioning and nutrient utilization, metabolism needs to be tightly linked for efficient growth. Previously, it had been determined that enhanced growth might be achieved through selection for individuals with increased appetite or who demonstrate a greater efficiency in partitioning energy from nutrients (Hill et al., 2000; Silverstein et al., 2000; Johnson et al., 2003). To understand the effects of selective breeding and transgenesis on these processes, expressions of the genes *IGF-I, IGF-II, fox01*, and fructose 1,6 bisphosphatase (*F1,6 Bp*) have been examined in muscle and liver. IGF previously has been studied in fishes by researchers interested in growth interactions involving the somatotropic axis (Pierce et al., 2001; Gomez-Requeni et al., 2003, Raven et al., 2008). Although IGF-I is known for its direct role in increasing animal size through the somatotropic axis, it also has been shown to stimulate growth by suppressing the expression of *atrogin-1* and *MuRF1* (Sacheck et al., 2004). IGF-II belongs to the same family as IGF-I and also is involved in the regulation of metabolism, growth, and differentiation (Planas et al., 2000). Also analyzed for expression were the IGF-I receptor 1a, whose expression has been correlated with IGF levels (Greene and Chen, 1999; Gabillard et al., 2003), and IGF-I binding protein 1, one of at least six IGF-binding proteins known in salmonids (Kamangar et al., 2006). The expression of other relevant metabolic genes analyzed included pyruvate kinase, pyruvate carboxykinase, pyruvate dehydrogenase, glutamate dehydrogenase, and aspartate aminotransferase.

Fox01 (forkhead box 01) is a member of a family of forkhead box transcription factors in muscle and can induce the muscle-specific ubiquitin ligases atrogin-1 and MuRF-1 which are known to participate in muscle degradation (Sandri et al., 2004; Lecker et al., 2004; Lang et al., 2006). In the liver of mammals, fox01 acts to regulate lipid and glucose metabolism (Valenti et al., 2008; Kamgate and Dong, 2008). Fructose 1,6 bisphosphatase is found mainly in the liver, where it functions in gluconeogenesis by converting smaller substrate molecules into glucose. Prior studies have demonstrated changes in the expression for this gene in the muscle of rainbow trout under different feeding conditions (Johansen and Overturf, 2006). Further, *REDD-1* expression in the liver also was measured. *REDD-1* is involved in regulating mTOR (mammalian target of rapamycin) activity and protein synthesis in mammals and in turn is regulated to some degree by ubiquitin ligases (Katiyar et al., 2009). Recent studies have shown the TOR pathway to be important in regulating protein accretion and growth in skeletal muscle in fish (Seiliez et al., 2008b). Another gene found to be linked with metabolism is the peroxisomal proliferator-activated receptor gamma (*PPARγ*) which has been linked with metabolic regulation of energy from incoming nutrients, particularly those involved with fatty acid metabolism (Kennedy et al., 2006; Leaver et al., 2008).

Degradation factors studied in both the liver and muscle in the present study include cathepsin L, calpain 1, 20S proteasome, and the long and short forms of calpastatin (*cast L* and *cast S*). Also included was the analysis of two muscle ubiquinase genes, *MuRF1* and *atrogin-1*. Regulation of the degradation system has been reported as crucial in regulating protein and muscle accretion, particularly in starved fish (Martin et al., 2001; Houlihan et al., 1988). There are four major systems involved in protein degradation, including calpains, cathepsins, proteasome and apoptosis. However, the first three have been shown to be the primary systems involved with maintaining and regulating metabolic and growth homeostasis. Calpains are a family of calcium-dependent, non-lysosomal cysteine proteases. They have been shown to be active in processes such as cell mobility, cell cycle progression, cell fusion in myoblasts, cell degradation, and muscle protein turnover and growth (Huang and Forsberg, 1998). Previous research has characterized the isoforms and catalytic subunits for μ and m calpains in rainbow trout (Goll et al., 2003; Salem et al., 2005a). Cathepsins are a family of cysteine and aspartic proteases that are distinguished by their structure and substrate specificity. They function at low pH and are almost exclusively found in lysosomes. In mammalian systems, cathepsins have been shown to play a vital role in cellular turnover and bone resorption (Yasuda et al., 2005). In salmonids, cathepsin has been identified as a protein whose abundance is increased in the liver during short-term starvation (Martin et al., 2001). Multiple studies also have recognized that changes in the regulation and activity of proteasome proteolysis are important for protein turnover in fish at different life stages and on different diets or feeding regimes (Seiliez et al., 2008a; Martin et al., 2002; Dobly et al., 2004; Overturf and Gaylord, 2009). In this study, the expression of *calpain1* (μ calpain), *cast S* and *cast L* was measured as an indication of calpain activity, cathepsin L for lysosomal degradation, and the proteosomal subunit proteasome 20S for evaluation of ubiquitin proteasomal degradation activity. *MurF1* and *atrogin-1* are ubiquitin ligases that have been linked to many areas including metabolism, but their major role has been depicted as factors involved with myofibril breakdown and muscle atrophy (Glass, 2003; Lecker et al., 2004; Hirner et al., 2008).

There has been some evidence of immunological changes in transgenically modified fish (Guo et al., 2003; Wang et al., 2006). Reduced resistance to *Vibrio anguillarum* was found for GH-transgenic coho salmon at certain life stages (Jhingan et al., 2003). In the present study, potential differences in the innate immune system between transgenic and non-transgenic coho salmon were evaluated by measuring the expression of complement factor C3, *TNFα*, *CXCL-8*, and

NFκβ. Complement factor C3 is a component of the innate immune system and has multiple functions, including downstream activation of the complement cascade and binding to pathogen surfaces to facilitate phagocytosis (Medzhitox and Janeway, 2000). TNFα (tumor necrosis factor α) is a cytokine that functions in systemic inflammation and can regulate other immune cells. TNFα also has been shown to inhibit tumor development and viral replication and to induce apoptosis and cellular inflammation. More recently, TNFα also has been found to be involved in metabolic regulation, including glucose signaling, regulation of plasma triglycerides and body fat in mammals (Ventre et al., 1997; Saltiel and Kahn, 2001). CXCL8 (interleukin 8) is a chemokine produced by macrophages that also function during an inflammatory response. CXCL-8 is secreted by several cell types and its primary function is to recruit neutrophils for phagocytosis of antigen (Gangur et al, 2002). NFκβ is a rapid-acting transcription factor that responds to stimuli, such as stress, cytokines, free radicals and antigenic pathogens. NFκβ functions as a first responder to harmful cellular stimuli, and over-activation can lead to a number of different inflammatory diseases (Gilmore, 2006). Other factors studied, such as glutathione peroxidase, which have a known role in protecting cells from oxidative damage, also were tested for evaluating potential stress effects from the different treatments (Otto and Moon, 2009). The glutathione system has been shown to be up-regulated in GH-transgenic salmon (Leggatt et al., 2007), but whether such effects are influenced at the gene regulation level is not well known.

Prior research has suggested similarities in gene expression at a global level between domesticated fish that have been selected for growth for several generations and fish that have undergone transgenic modification for increased production of growth hormone (Devlin et al., 2009a). To examine in detail specific pathways involved, real-time PCR analysis was used to assess the expression of genes involved in metabolism, muscle development and growth, and immune function. Expression was assessed in wild-type, domesticated and GH-transgenic coho that have been either fully-fed or feed-rationed to discern some of the more specific metabolic and other physiological differences generated by either domestication or GH transgenesis. Further, assessment of expression of the different gene panels used in this study will improve our knowledge of how increased growth hormone production is translated into animal growth and its effect on muscle accretion. Additionally, with the use of appropriate controls, effects such as domestication, GH-transgenic manipulation and available energy can be determined so that an enhanced understanding of the pathways and mechanisms involved with metabolism and growth can be better understood and used to improve selection or enhance future transgenic models.

2. Materials and methods

2.1. Animal care and husbandry

Strains of coho salmon (*Onchorhynchus kisutch*) were grown within the Transgenic Fish Facility at the Fisheries and Oceans Canada Centre for Aquaculture and Environmental Research (CAER) in West Vancouver, Canada. This bio-contained facility is designed to prevent the escape of fish to the natural environment. Fish were reared under an approved Animal Use Protocol in accordance with the Canadian Council on Animal Care Guidelines. Experimental fish were selected from families generated by artificial fertilization of eggs (10 single-pair matings per genotype). Gametes from wild-strain salmon were obtained from the Chehalis River in southwestern British Columbia in fall 2004, and gametes from domesticated coho salmon mass-selected for growth were obtained in November 2005 from a British Columbia aquaculture producer. Briefly, this growth-enhanced domesticated strain was originally derived from Kitimat River and Roberson Creek populations and was selected for enhanced growth for six generations. Substantial anecdotal information from aquaculture and laboratory observations, in addition to published data described by Devlin et al. (2001, 2009b) and Tymchuk et al (2006), suggest that this domesticated strain grows faster than native wild coho salmon populations. Hemizygous growth hormone-transgenic salmon were generated in January 2006 by matings between GH-transgenic strain M77, F_6 generation, (Devlin et al., 1994, 2004) and Chehalis River wild-type salmon. Utilizing previous knowledge of these fish, including growth histories and spawning times (Tymchuk et al., 2006; Raven et al., 2006; Devlin et al., 2001), size-matched individuals were generated for this study. Ten fish from each group were used for analysis. Weights and total lengths were taken for each fish, and specific growth rates were calculated (% growth/day). Fish were reared in 10 °C fresh well water and fed artificial salmon diets (Skretting Canada) to satiety three times per day for the domesticated, wild-type and transgenic full-fed groups. At each feeding, ration-restricted GH-transgenic salmon (Tran-RR) were pair-fed to the ration level consumed by the wild-type strain.

2.2. Sampling and RNA isolation

Fish became size- and stage-matched in October 2006. The fish were not fed on the day when samples were taken and before euthanization (100 mg/L of MS222 plus 200 mg/L sodium bicarbonate). Tissues from ten fish from each group were removed rapidly from individual fish by team dissection and placed into RNAlater (Ambion, Austin, TX) following the manufacturer's instructions. RNA isolations were carried out using TRIzol according to the manufacturer's protocol (Invitrogen, Rockville, MD). Tissues were homogenized by adding a 5 mm stainless steel bead to the RNA-TRIzol mix and then shaking twice for 2 min at 40 Hz using a Qiagen MM301 shaker (Valencia, CA). RNA from each fish was DNAse-I treated (Promega, Madison, WI). Quantity and quality checks of the RNA were done by spectrophotometry.

2.3. Quantitative real-time RT-PCR and data analysis

Quantitative PCR (QPCR) analyses were performed on 10 fish for each experimental treatment (wild-type, domesticated, GH-transgenic full-fed, and GH-transgenic feed-restricted). Primers and probes were designed either with Primer Express 3 software or by Applied Biosystem Assay by Design (ABI, Foster City, CA). To detect the level of gene expression at each time-point, real-time quantitative RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI, according to the protocol provided by ABI (Foster City, CA). The final concentration of each reaction was: Master Mix, 1x (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U/μL; RNase inhibitor mix, 0.4 U/μL; forward primer 600 nM; reverse primer 600 nM; probe, 250 nM; total RNA, 75 ng with water added for a total reaction volume of 15 μL. Probes and primer sequences are listed in Table 1. Cycling conditions for genes tested were as follows: 30 min at 48 °C, 10 min at 95 °C, then 40 cycles of PCR consisting of 15 s at 95 °C followed by 1 min at 60 °C. For each gene, assays were run in duplicate on RNA samples isolated from individual fish.

Relative copy number for the expression of each gene tested was determined by serially diluting a random experimental sample 10-fold and including this in each set of real-time assays run. The data are reported as a ratio of relative mRNA copy number of each gene to absolute mRNA copy number of β-actin in liver (Kreuzer et al., 1999) or 18S ribosome in muscle (multiplied by a constant for ease of interpretation) and expressed as means ± standard errors. Microsoft Excel was used to produce graphical representations of the data. One-way ANOVA and *t*-test analysis of the data were performed using SYSTAT ver. 9 (SPSS, Chicago, IL). One-way ANOVA with post-hoc testing by Student–Newman–Keuls was used for comparison analysis of fish growth.

Table 1
Genbank accession numbers and sequences of primers and probes used for real-time PCR analysis.

Gene	GenBank/TIGR accession no.	Primer-probe sequence (5′–3′)
18S rRNA	AF243428	18SF: CCACGCGAGATTGAGCAAT 18SR: GCAGCCCCGGACATCTAA 18SPR: 6FAM-ACAGGTCTGTGATGCC-NFQ
Atrogin1	BX082508	ATRF: CCACTCAGGACATCCAGAGATTTAC ATRR: CAGCTGAGACCTGGCTATGAG ATRPR:6FAM-CTATGTGGCAAAACTC-NFQ
Aspartate aminotransferase	TC9251	AATF: CATGCCTGTGCCCATAACC AATR: CACCAGGTCAGCGATCTCTTT AATPR: 6FAM-CCACTGCTCAGGCTTG-NFQ
β-Actin	AF254414	BactF: CCCTCTTCCAGCCCTCCTT BactR: AGTTGTAGGTGGTCTCGTGGATA BactPR: 6FAM-CCGCAAGACTCCATACCGA-NFQ
Calpain1	AY573919	Calp1F: CCAGGGCCAGGACTTTAAGT Calp1R: AGGCGCACGGGAACAG Calp1PR: 6FAM-CCGCAGCCTGTTTGAG-NFQ
CalpastatinL (cast L)	AY937407	CastLF: GCTCCAGCTGTCCATGCT CastLR: GCATCCAAGGCAAAGTCATCTGA CastLPR: 6FAM-CCCCAGCTCCTCCC-NFQ
CalpastatinS (cast S)	AY937409sd	CastSR: AGCTGTCCAATCCTGTGAGAAAA CastSR: TGAAGCAACATCACTGCAACATTG CastSPR: 6FAM-CTAGGGCAACACCTCCATTT-NFQ
CathepsinL	AF358668	CathLF: CAGTGCTGCCAACGAGACT CathLR: GCCTTCATCATAGCATGCTCCTT CathLPR: 6FAM-CTTTGTGGACATCCCC-NFQ
Complement C3	AF271079	C3F: CATTGGCCTGTCCAAAACACAT C3R: GCAGCTTCAGATCAAGGAAGAAGTT C3PR:6FAM-CAGACACACAGATTCC-NFQ
Conserved edge expressed	EF036472	*cee*F: CGCAGCGAGGTGGACAT *cee*R: TGTTCTTTAGACAGAGGAACTGTAGGA *cee*PR: 6FAM-CCTGCGCCACAAAC-NFQ
CXCL8	AJ279069	IL8F: AGCCAGCCTTGTCGTTGTG IL8R: AGTTTACCAATTCGTCTGCTTTCC IL8PR: 6FAM-TCCTGGCCCCTCCTGACCATTACTGAG-TAMRA
Fox01	CX247152	Fox01F: CGCTGGTGGCAGTGGT Fox01R: TCCAGGTCAGTGGGAAAGC Fox01PR: 6FAM-CCTAGCGAGCAGATTC-NFQ
Fructose 1,6-bisphosphatase	AY113693	FR16BPF:CGTTATGTCGGCTCCATGGT FR16BPR: TGCCTCCGTACACCAGAGT FR16BPPR: 6FAM-CCTGTGCACATCAGGC-NFQ
Glutamate dehydrogenase	TC9373	GDHF: GAGCAGAAGATGAAACGAGTGAAG GDHR: CTCTCCGTTGTCCCTCTTGATG GDHPR: 6FAM-AAGCCCTGCAACCAC-NFQ
Glutathione peroxidase	AF281338	GPF: CCTCCTCCTCCTCACAAGCT GPR: CAGGAGAGCACGGTACATGATTTAA GPPR: 6FAM-ATGGGTGCTGTGGTCACA-NFQ
IGF-I	M95183	IGF1F: GTGTGTGGAGAGAGAGGCTTTTATT IGF1R: CGTCCACAATACCACGGTTATGT IGF1PR: 6FAM-ATGGCCCCAGTTCACG-NFQ
IGF-II	M95184	IGFIIF: GAGGCGTGGCAGAGGAA IGFIIR: GCCTGCCTCCGGAACTT IGFIIPR: 6FAM-TCCCGGCCATCCTC-NFQ
IGF-I receptor 1a	AY100459	IGF1r1aF: GAAGGAGAGCCCCCCTGAGA IGF1r1aR: CAGTGAAGGGCTGCAGGTT IGF1r1aPR: 6FAM-CTTGCTCCGGTCCTCC-NFQ
IGF-I binding protein1	DQ190460	IGFbpF: CACAGCTCCCTAGCCTACTTC IGFbpR: CTCTCCTGAGCACCCTCATTTT IGFbpPR: 6FAM-CTGGGACTCAACACCC-NFQ
MEF2A	CA374878	*MEF2A*F: GGCCAGGCAGCTCTCA *MEF2A*R: TTGGAGCCCTGAGGTAGGT *MEF2A*PR:6FAM-CACCCACCAAAGAGC-NFQ
MEF2C	CA380324	*MEF2C*F: CCCTAGGCAACCACAACCT *MEF2C*R: ACTGGGAGGTCTATGTGTGACA *MEF2C*PR: 6FAM-CCGTCCCATGACCCC-NFQ
Myf5	AY751283	*Myf5*F: CACAAGCTATGGCAACAACTACAG *Myf5*R: GGCACCAGCACCTCTCT *Myf5*PR: 6FAM-TCCAGAGCTCACATTCT-NFQ
MuRF1	BX319947	MuRF1F:CGCAATCCCTACCGCTTCTC MuRF1R:GGTCCAGGACCACCTCGAA MuRF1PR:6FAM-TCCGCTGCCCCACCTG-NFQ
NFκβ	CA356763	NFKBF: TGACAAAGGCATCTGCATCACA NFKBR:CATGGAGGATGCCCAGGTT NFKBPR:6FAM-CTGATGCTGGAGTCTTT-NFQ
Pax7	CB493668	*PAX7*F: CTATGTGGCAAAACTC *PAX7*R: GTCCCAGCATGACTTCTCCAT *PAX7*PR: 6FAM-CAGCCTGGAGTCCTC-NFQ

Table 1 (*continued*)

Gene	GenBank/TIGR accession no.	Primer-probe sequence (5′–3′)
PPARγ	AJ292963	PPARgF: AGGCCATCCTCTCTGGAAAGA PPARgR: CGTCAGAGACTTCATGTCATGGAT PPARgPR: 6FAM-CCCACGGAAACTCAC-NFQ
Proteasome 20 Δ	AF115539	ProtΔF: GAGGGTCAGGATCCACCTATATCTA ProtΔR: GCGAAGACACTGGTCCTTTGT ProtΔPR: 6FAM-ACTCCAACTACAAACCC-NFQ
Pyruvate carboxylase	TC105924	PCF:GCCCTGTAGACTGCCTCAA PCR: CTCTTCAGCAATCTTGGGTCCAT PCPR: 6FAM-CACACGCCTGTTCCTC-NFQ
Pyruvate dehydrogenase	TC28238	PDH2F: GTAGTGAGGTCCCAATGTCATACTT PDH2R: TGGGCACAGTATCTGAGTCTTCA PDH2PR: 6FAM-CTGCCACATCTCTCCC-NFQ
Pyruvate kinase	AY113695	PKF: ACAGCGTGGGCGATACC PKR: GCTGGAGCTGTCATAGTACTCACT PKPR: 6FAM-TCAGCCCAGCTCCTG-NFQ
T*MyoD*2	Z46924	*MyoD*F: GCCGTCACCGACCAACT *MyoD*R: CACTGTGTTCATAGCACTTGGTAGA *MyoD*PR: 6FAM:CCGTCCCATGACCCC-NFQ
T*myogenin*	Z46912	*Myogenin*F: CATGGACCGGCGGAAAG *Myogenin*R: GGCCTCGAATGCCTCGT *Myogenin*PR : 6FAM-CTTCTTCAGCCTCCTCTT-NFQ
Tmyostatin1	AF273035	TMSTAT1F: CCGCCTTCACATATGCCAA TMSTAT1R: CAGAACCTGCGTCAGATGCA TMSTATPR: 6FAM-CATATTACATTTGGGATTCAA-NFQ
Tmyostatin2	AF273036	TMSTAT2F: AGTCCGCCTTCACGCAAA TMSTAT2R: ACCGAAAGCAACCATAAAACTCA TMSTAT2PR: 6FAM-CGTATTCACTTTTGGATTTT-NFQ

3. Results

3.1. Growth rates and relative sizes of fish

Size-matched fish from the domesticated, wild-type, full-fed (Tran-FF) and restricted ration (Tran-RR) growth hormone-transgenic coho salmon were used for analysis in this study. Obtaining size and weight-matched individuals was possible due to existing knowledge regarding their spawning times and growth history (Tymchuk et al., 2006; Raven et al., 2006; Devlin et al., 2001). No significant difference in weight was observed between the four groups. Wild-type and Tran-RR fish were significantly greater in total length than domestic and Tran-FF fish, although the difference was slight (Fig. 1). Specific growth rates (SGR) for both weight and length were greatest for Tran-FF fish, significantly more so than for domesticated fish, which were significantly greater than those for wild-type and Tran-RR fish. There was no significant difference in SGR between wild-type and Tran-RR fish for either weight or length.

3.2. Gene expression

3.2.1. Muscle gene expression

The expression of twenty-one genes was evaluated in the muscle of the experimental transgenic and non-transgenic coho salmon. The genes are divided into the following groups; muscle propagation, muscle development and regulation, muscle degradation, and muscle metabolism.

Muscle propagation factors analyzed included *Pax7* and *cee*. The expression of the *Pax7* gene was significantly higher in the muscle of domesticated fish compared to wild-type, Tran-FF and Tran-RR salmon. There was no significant difference between the latter three groups. The expression of the *cee* gene significantly increased in the muscles of Tran-FF fish compared to the other experimental treatments, among which no significant difference was detected (Fig. 2).

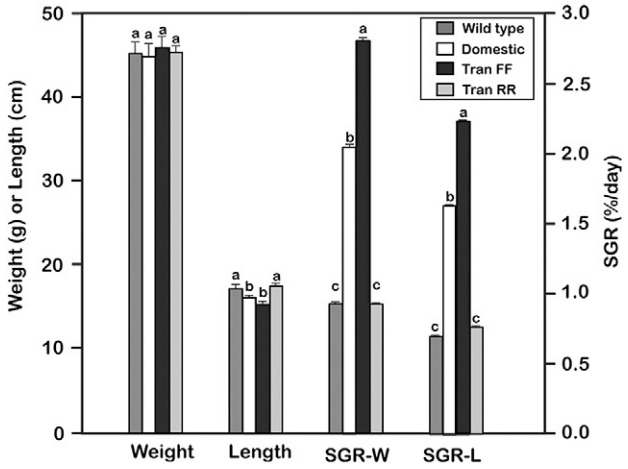

Fig. 1. Weight, total length, and SGR (specific growth rate, % daily for length (*L*) and weight (*w*) data for wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone-transgenic coho salmon. Bars within a category on the *X*-axis that do not share identical lowercase letters are significantly different when compared among groups at $P<0.05$.

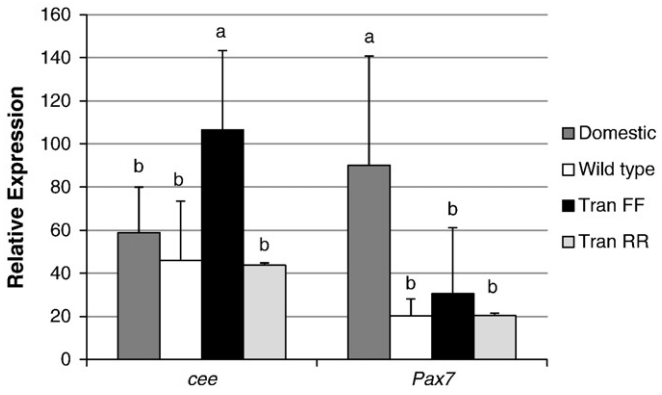

Fig. 2. Expression of muscle differentiation and propagation factors *cee* (conserved edge expressed protein) and Pax7 in the muscle of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$.

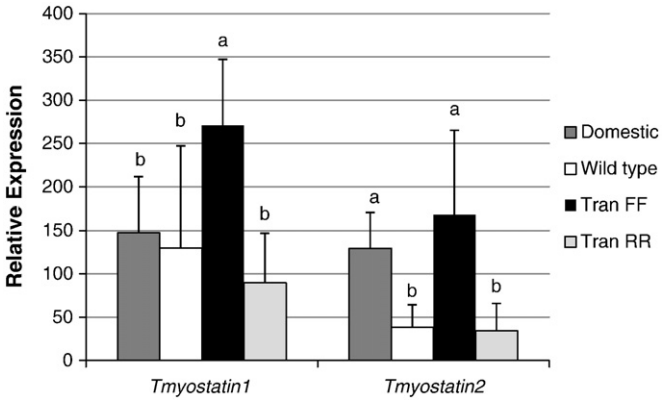

Fig. 3. Expression of myostatin genes in the muscle of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$.

Muscle developmental and regulation factors evaluated included *myostatin 1* and *myostatin 2*, *myoD2*, *Myf5*, *mef2A*, *mef2C*, and *myogenin*. The relative expression of *myostatin 1* and *2* was the same, with the highest expression found in Tran-FF salmon. For *myostatin 1*, no significant differences were found between the other three experimental groups. However, for *myostatin 2*, Tran-FF and domesticated coho salmon expression levels were not significantly different, but both were significantly higher than those for wild-type or Tran-RR fish (Fig. 3). Expression of *myoD2* was significantly different between all four groups, with Tran-FF fish showing the highest expression level, followed by domesticated fish, then Tran-RR, and followed by wild-type coho salmon which had the lowest expression level. Relative *Mef2A* transcription levels were similar to those found for *myoD2* in that significant differences were found between all the groups, with Tran-FF showing the highest level of expression followed by domesticated, Tran-RR, and wild-type coho salmon. *Myogenin* expression was significantly higher in FF-transgenic fish, followed by domesticated coho salmon and Tran-RR between which there was no significant difference, and both were significantly higher in expression than wild-type fish. Expression of *myf5* was similar and significantly higher in domesticated and Tran-FF salmon compared to Tran-RR and wild-type fish. *Mef2C* expression was not significantly different between domesticated, wild-type and Tran-RR salmon; however, the expression of *mef2C* was significantly higher in domesticated than in Tran-FF fish (Fig. 4).

Muscle degradation genes analyzed for expression consisted of the short and long forms of calpastatin (*cast S* and *cast L*), *calpain 1*, *cathepsin L*, and the proteosomal subunit 20S delta. Expressions of the ubiquitin ligases *MuRF-1* and *atrogin-1* also were evaluated. Expression of the proteasome delta 20S subunit was significantly higher in the muscle of Tran-FF fish than in domesticated fish which was significantly greater than that for wild-type and Tran-RR fish, among which there was no significant difference. *Calpain 1* was significantly up-regulated in the muscle of domesticated fish relative to other experimental groups which showed no significant differences among one another. The calpain inhibitors *cast S* and *cast L* were similarly expressed in all four groups, with wild-type and Tran-RR fish similarly showing the lowest expression. Expression was highest in domesticated fish, significantly higher in the case of *cast S*, but not *cast L*. For both *cast S* and *cast L*, expression was significantly greater for the domestic and Tran-FF fish compared to wild-type and Tran-RR fish. *Cathepsin L* expression differed significantly between all the groups, with wild-type coho salmon having the highest expression, followed by Tran-RR then domesticated and Tran-FF fish. For the ubiquitin ligases, *MuRF1* expression was similar for domesticated, wild-type and Tran-RR fish, but expression was strongly (>12-fold) and significantly reduced in Tran-FF fish. *Atrogin-1* gene expression was significantly higher in domesticated and Tran-FF fish, with no difference between these groups. There was no significant difference between the lower- expressing wild-type and Tran-RR fish groups (Fig. 5).

Genes associated with metabolism and growth whose expression were examined in muscle consisted of *IGF-I*, *IGF-I bp1*, *IGF-II*, *fox01* and fructose 1,6 bisphosphatase. *IGF-I* expression was elevated in fast-growing Tran-FF and domestic genotypes relative to wild-type and Tran-RR fish, with the highest expression in Tran-FF fish and domesticated coho salmon significantly lower (>3-fold). There was no significant difference between wild-type and Tran-RR fish, which both expressed *IGF-I* at levels significantly less than for domesticated coho salmon. Expression of the binding protein *IGF-I bp1* was highest in Tran-RR fish, although not significantly greater than for domesticated fish. However, for both Tran-RR and domesticated fish, expression levels were significantly greater when compared to Tran-FF fish. There was no significant difference in expression between domesticated and wild-type fish for this receptor. No significant differences were found for *IGF-II* expression between all four groups. Expression of *fox01* was significantly higher in both domestic and Tran-FF when compared to wild-type and Tran-RR fish. No significant difference in expression was found between domestic and Tran-FF or between wild-type and Tran-RR fish. Expression of *F1,6 Bp* showed no significant difference between

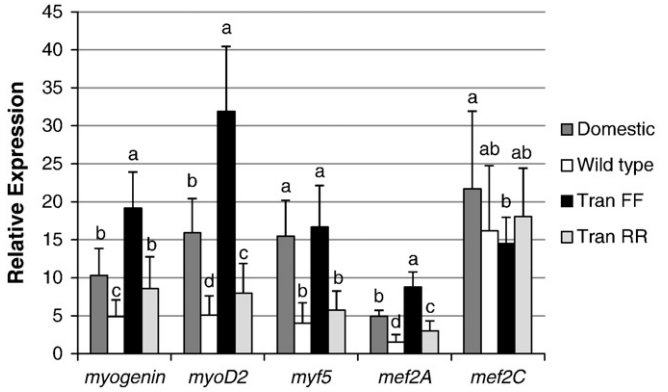

Fig. 4. Expression of muscle regulatory factors in the muscle of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$.

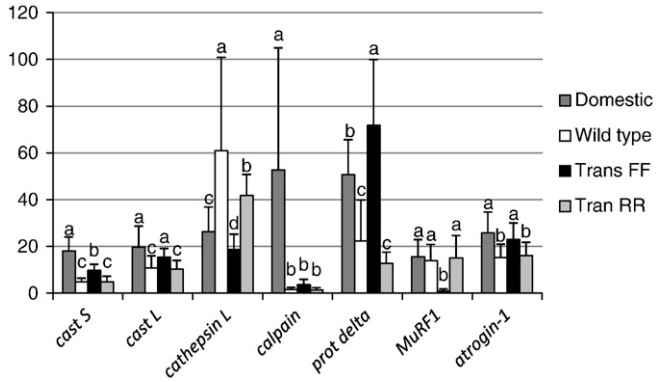

Fig. 5. Expression of degradation factors in the muscle of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$.

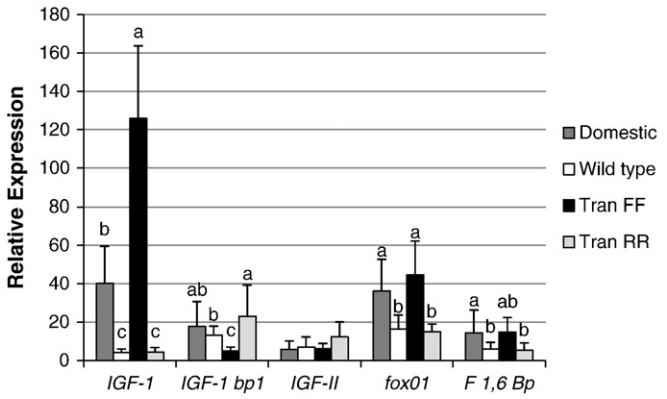

Fig. 6. Expression of metabolic-related factors in the muscle of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$. (*IGF1a rec- IGF-I 1a receptor, F1,6 Bp- fructose 1,6 bisphosphatase*).

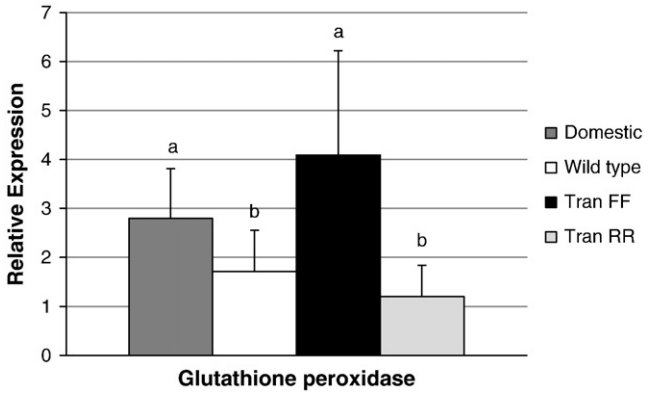

Fig. 8. Expression of glutathione peroxidase in the liver of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. Bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$.

domesticated and Tran-FF fish. However, domesticated fish did show significantly higher expression than wild-type and Tran-RR fish (Fig. 6).

3.2.2. Liver gene expression

The expression of sixteen genes was evaluated in the liver of transgenic and non-transgenic coho salmon. The genes were selected for analysis based on their involvement with immunological response, cellular damage response, growth factors, and/or energy metabolism.

The immunological genes analyzed for expression were complement factor C3, tumor necrosis factor α (*TNFα*), *CXCL-8* (IL-8) and *NFκβ*. Complement factor C3 was significantly up-regulated in the liver of FF transgenic fish when compared to the other treatments; however, among the other three treatments there was no significant difference. *TNFα* expression showed no difference between wild-type, Tran-FF, and Tran-RR, and the expression for all these groups was significantly higher than for that of the domesticated fish. *CXCL-8* expression was significantly different between all groups, with the expression in Tran-FF highest, followed by Tran-RR, wild-type, and domesticated coho salmon. *NFκβ* expression was highest in domesticated fish, and while it was significantly higher than the expression for Tran-FF and Tran-RR fish, it was not significantly higher when compared to wild-type coho. There was no significant difference in the expression of *NFκβ* between wild-type, Tran-FF and Tran-RR fish (Fig. 7).

Glutathione peroxidase expression was examined to determine whether there were any changes involving oxidative damage protection. Expression of this gene was significantly higher in Tran-FF and domesticated compared to Tran-RR and wild-type fish, with no significant difference between these latter two groups (Fig. 8).

Growth factor genes examined in the liver included *IGF-I*, IGF-I receptor 1a (*IGF-I rec1a*), *IGF-II* and *fox01*. The expression of *fox01* was significantly higher in the liver of domesticated coho salmon than for the other three groups, among which no significant difference in expression was determined. *IGF-I* expression was highest in domesticated fish, with no significant difference between these fish and Tran-FF fish. There was no significant difference between Tran-FF and Tran-RR groups, with expression for both of these groups significantly higher than for wild-type coho salmon. For *IGF-I rec1a*, wild-type fish exhibited highest expression, which while being more than 30% over that for Tran-RR, was not significantly greater. Both wild-type and Tran-RR expression of *IGF-Ibp* showed significantly higher levels of expression than domesticated fish. Tran-FF fish expression of *IGF-Ibp* was significantly lower than the expression observed for the other three groups. Expression of *IGF-II* was found highest in the liver of domesticated fish, followed by Tran-FF, where it was significantly less. Wild-type and Tran-RR fish had significantly lower expression of IGF-II compared to domesticated and Tran-FF fish, but there was no significant difference in expression between these two groups (Fig. 9).

Expressions of seven energy metabolism-related genes also were evaluated in the livers of these groups of coho salmon. These genes included pyruvate dehydrogenase (*PyrD*), pyruvate carboxylase

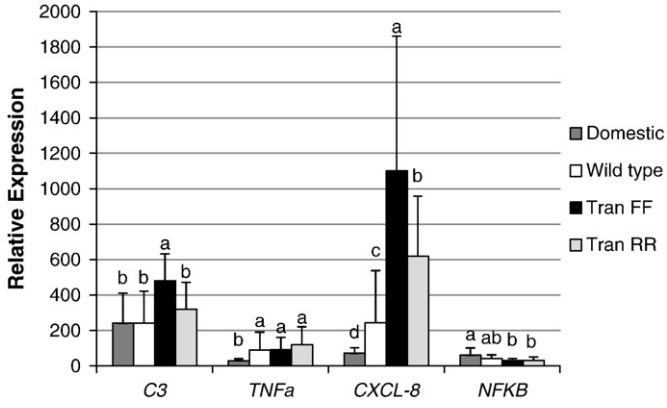

Fig. 7. Expression of immunological factors in the liver of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$. (*C3* — complimentary component C3, *TNFα* — tumor necrosis factor α, *CXCL-8*).

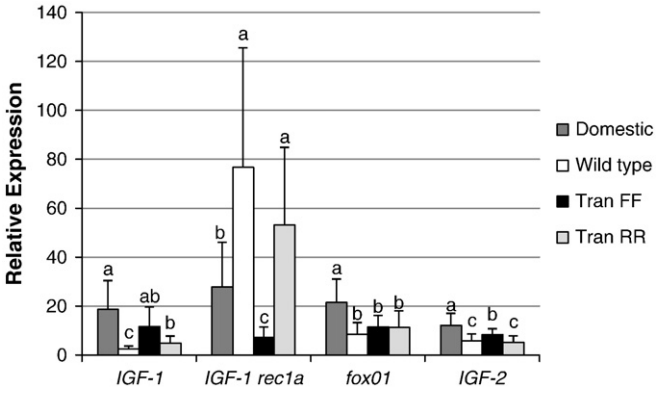

Fig. 9. Expression of genes related to growth in the liver of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$.

(*PyrC*), pyruvate kinase (*PK*), glutamate dehydrogenase (*GlutD*), aspartate aminotransferase (*AspAm*), *PPARγ*, and fructose 1,6 bisphosphatase (*F1,6Bp*) . Expression of *PyrD* was highest in Tran-FF fish. Expression of *PyrD* did not differ between domesticated and Tran-FF fish, but both expressed *PyrD* at levels significantly higher than those found for wild-type and Tran-RR fish; there was no statistical difference in expression between wild-type and Tran-RR fish. *PyrC* was expressed in domesticated coho at significantly higher levels than in Tran-RR, which expressed this gene at levels significantly higher than those found for wild-type and Tran-FF coho salmon. There was no significant difference detected for *PyrC* between wild-type and Tran-FF fish. *PK* was expressed highest and approximately equally in the two transgenic groups. While expression of *PK* was significantly higher in the Tran-FF fish than in the domesticated fish, there was not a significant difference in expression between the Tran-RR and the domesticated coho salmon. Wild-type fish expressed *PK* at a significantly lower level than in other groups. The enzyme glutamate dehydrogenase was similarly expressed in domesticated, wild-type, and Tran-RR fish, only showing significant reduction in expression in the liver of Tran-FF fish. Aspartate aminotransferase was expressed at similar higher levels in domesticated and Tran-FF fish as than in wild-type and Tran-RR coho fish, where no significant difference was noted between either domesticated and Tran-FF fish or wild-type and Tran-RR fish. Similarly, expression of *PPARγ* was highest in Tran-FF and domesticated fish, with no significant difference between these two groups. However, in the Tran-RR group *PPARγ* was expressed at significantly lower levels than in domesticated and Tran-FF fish. Wild-type fish expressed *PPARγ* at a significantly lower level than the other three groups. Unlike in the muscle, where fructose 1,6 bisphosphatase was expressed highest in the Tran-FF fish, in the liver this enzyme was expressed at the lowest level in Tran-FF, but not in a manner that was statistically different than in domesticated and wild-type fish. In this case, Tran-RR was found to express the *F1,6 bp* gene at a significantly higher level than in the other experimental groups. *REDD-1* expression was highest in Tran-FF coho salmon, where it was significantly higher than in wild-type and Tran-FF fish, among which no difference was detected. Domesticated coho salmon were found to express *REDD-1* at a significantly lower level than the three other groups (Fig. 10).

4. Discussion

A number of fish species have been genetically engineered to enhance the expression of specific growth factors, primarily growth hormones (Du et al., 1992; Rahman and McLean 1997; Devlin et al., 1994, 2001). Results have shown that when wild-type salmonids are transgenically modified to express greater levels of growth hormone, relative body sizes at specific ages can increase up to 37-fold (Devlin et al., 1994). However, transfer of GH genes into domesticated fish that have been prior-selected for growth (Tymchuk et al., 2006) showed a reduced growth response to GH (Devlin et al., 2001). Similar attenuation of GH effects also were seen in domesticated Atlantic salmon and rainbow trout treated with GH protein (Devlin et al., 2001; Neregard et al., 2008). These data are consistent with the hypothesis that selection for faster-growing fish is mediated by increased stimulation of the growth hormone signaling pathway. From prior experiments, it appears that the answer is yes and no. That is, the level of growth hormone is higher in faster-growing animals, but certain physiological and molecular differences have been noticed between the transgenic and selected domesticated fish (Devlin et al., 2001). The research described herein showed several correlations in the expression of different genes for the fish that grow the fastest, domestic selected and Tran-FF coho, versus the smaller fish, wild-type and Tran-RR coho salmon. However, the expression of some genes was quite divergent between full-fed transgenic fish and domesticated coho salmon.

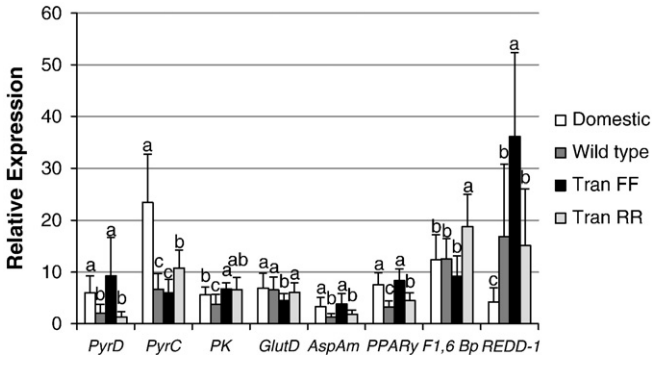

Fig. 10. Expression of metabolic-related genes in the liver of domesticated, wild-type, and full-fed (Tran-FF) or restricted-rationed (Tran-RR) growth hormone transgenic coho salmon. For each gene, bars that do not share identical lowercase letters are significantly different when compared among fish groups at $P<0.05$. (*PyrD* — pyruvate dehydrogenase, *PyrC* — pyruvate carboxylase, *PK* — pyruvate kinase, *GlutD* — glutamate dehydrogenase, *AspAm* — aspartate aminotransferase, *PPARγ* — peroxisomal proliferator-activated receptor γ, *F1,6 Bp* — fructose 1,6 bisphosphatase).

4.1. Muscle

Analysis of muscle gene expression revealed both similarities and differences regarding expression of genes whose products play a role in the muscle physiology of wild-type, domesticated and transgenic coho salmon. The muscle development factors *cee* and *Pax7* both showed significant differences among Tran-FF and domesticated fish. Tran-FF coho salmon exhibited up to a 2.5-fold increase in the expression of *cee* in muscle compared to the other experimental groups. The *cee* gene originally was isolated from genome screens seeking genes associated with myotube formation (Fernandes et al., 2005). In fugu, *cee* was up to 15-fold up-regulated in fast-twitch muscle and relatively unchanged in other tissues. In salmonids, *cee* is expressed in developing organs and tissues (Macqueen et al., 2007). The increased expression of *cee* in Tran-FF salmon muscle during this study suggests that relative to the other treatments, when transgenic fish have sufficient nutrients available, they potentially have the ability to continually grow muscle in a hyperplastic fashion, consistent with the greater number of small fibers seen in GH-transgenic or GH-treated fish (Fauconneau et al., 1996; Hill et al., 2000). Unlike *cee*, *Pax7* was significantly up-regulated in the muscle of domesticated fish compared to wild-type and transgenic coho salmon. Pax genes are tissue-specific transcription factors that are important during development and have been associated with myogenesis and the developmental specification of satellite cells in the skeletal muscle in the presence of *myoD* (Zammit et al., 2006). *Pax7* functions through the regulation of the bHLH factor *Myf5*, whose expression was significantly greater for both domesticated and Tran-FF compared to wild-type and Tran-RR fish. These findings suggest that the enhanced muscle growth identified in the faster-growing fish is being modulated from multiple signals, beginning during differentiation and most likely continuing through developmental regulation and degradation of muscle tissue. Hence, while *cee* expression is higher in Tran-FF fish, *Pax7* expression was increased in domesticated fish. In other vertebrates, reduced *Pax7*/*MyoD* transcript ratios are known to be a transcriptional signature reflecting the enhancement of satellite cell myogenesis (Holterman and Rudnicki, 2005; Olguin et al., 2007). In this study transgenic fish overall had reduced ratios of *Pax7*/*MyoD*, average 0.021 for transgenic fish vs 0.071 for non-transgenic fish. However, downstream MRFs were all increased for domesticated and Tran-FF, with *MyoD*2 and *myogenin* significantly increased for Tran-FF fish. This finding would suggest that enhanced levels of growth hormone in salmonids can enhance satellite cell activation and differentiation. Since some of these changes were not observed in

Tran-RR, these responses are likely contingent upon availability of energy.

Regarding the other muscle regulatory genes evaluated, *myogenin* and *myoD*2 showed highest expression levels in Tran-FF, followed by domesticated, then Tran-RR, with wild-type coho salmon as the lowest-expressing group for these two genes. These results also were found for the expression of the muscle regulatory factor, *myf5*. Experiments in fishes have observed increased expression of *myoD* in faster-growing animals (Alves de Almeida et al., 2008; Wilkes et al., 2001). It is possible in fish that a level of hyperplasia is always present as they grow, and that this level is further affected by size and rate of growth of the individual.

Myocyte enhancer factor (MEF) family genes, which typically work together with MRFs to regulate muscle differentiation and growth but not specification, showed variable results. *Mef2A* has been shown to be crucial for maintaining appropriate mitochondrial content and architectural integrity in postnatal heart muscle in mice (Naya et al., 2002). *Mef2A* showed similar expression patterns as that seen for the MRFs, highest in Tran-FF and domesticated fish and lower in Tran-RR and wild-type fish. Regulation of differentiation and muscle development is regulated largely through heteroprotein complexes of MRFs and MEFs, which then control the transcription of muscle structural genes. Little change was detected for the expression of *mef2C*, suggesting that regulation is taking place through a mixture of MEFs and MRFs and that *mef2A* may be more directly involved. In all cases, it was noted that expression was highest in Tran-FF fish, followed by domesticated fish, further demonstrating how increased growth hormone level enhances muscle development. In most cases, it was found that in Tran-RR fish, expression of muscle factors was notably higher than those found for wild-type fish. This observation indicates that even with reduced available energy, growth hormone is essential for the allocation of available energy for muscle development and suggests that higher growth hormone levels can lead to more efficient feed utilization, as has been previously suggested (Raven et al., 2006; Oakes et al., 2007).

The relative expression of *myostatin 1* and *2* genes was not significantly different, which differs from previous results where *MSTN2* gene expression was reduced in transgenic compared to non-transgenic salmon (Roberts et al., 2004). Prior research has demonstrated that the age and current growth state of the fish when muscle samples are analyzed have a significant effect on the expression of myostatin and other genes (Johansen and Overturf, 2005; Levesque et al., 2007). In this study, expression of both myostatin genes was highest in Tran-FF fish. Furthermore, prior research has demonstrated that the expression of the myostatin genes is higher in larger, faster-growing fish (Johansen and Overturf, 2005). When energy is available, myostatin expression appears to be increased in faster-growing fish, but apparently is not negatively acting to regulate muscle growth. Thus, it would appear that energy availability influences muscle development more than repression by myostatin.

Expression of genes involved in regulating protein degradation in muscle samples showed similar responses for both the long and short forms of calpastatin, a gene which acts as a negative regulator of calpain. Both forms of calpastatin were significantly up-regulated in the muscles of domesticated and Tran-FF fish compared to wild and Tran-RR fish. However, even though domesticated fish displayed the highest level of *cast L* and *S* expression, the expression of *calpain1* was significantly up-regulated in the muscle of domesticated fish when compared to that in the other three groups. It is possible that calpastatin levels were just beginning to increase to compensate for the high calpain expression; sampling and evaluation at multiple time-points would be necessary to clarify any modulation of the regulation of these genes. Although calpain activity has been demonstrated to influence fillet quality, it is not believed to function substantially in active muscle turnover in salmonids (Salem et al., 2005b). For *cathepsin L*, expression was significantly higher in wild-type fish, followed by Tran-RR, domesticated, and finally Tran-FF fish. Cathepsin L plays a major role in the turnover of intracellular and secreted proteins involved in growth regulation (Kane and Gottesman, 1990; Ishidoh and Kominami, 1995). Although cathepsin L is recognized as a lysosomal protease, it also is secreted and therefore is potent in degrading extracellular and structural proteins (Mason, 1989). In wild-type coho salmon, cathepsin could be playing a role in increased protein breakdown and turnover linked to the relatively inefficient use of available energy in these fish. Expression of the ubiquitin proteasome pathway gene, delta subunit of proteasome 20, was expressed significantly higher in the faster-growing domesticated and Tran-FF fish. This pathway is believed to function as the main mode of muscle degradation (Hochstrasser, 1996; Ciechanover, 2005) and turnover in most vertebrate species and demonstrates that with enhanced protein accretion there also occurs increased muscle turnover.

Fox01 is a negative regulator of myogenesis and its induction of proteasome-dependent degradation of a specific subset of components in the mTOR signaling network of mammalian cells during differentiation impairs *IGF-II* expression (Wu et al., 2008). The activity of *fox01* is known to be controlled by its phosphorylation status, which was not determined in this study; however its regulatory actions occur mainly via modulation of gene transcription. Fox01 induces expression of both *atrogin-1* and *MuRF1*. Interestingly, *MuRF1* expression was strongly reduced in the muscle of Tran-FF fish compared to the other treatments. Past research has shown up-regulation of *MuRF1* and *atrogin-1* during muscle atrophy (Gomes et al., 2001) and down-regulation during age-related loss of muscle (Edstrom et al., 2006). Feed deprivation in salmonids and rats up-regulates expression of ubiquitin ligases; however, treatment with IGF-I prevents this induced up-regulation (Cleveland et al., 2009; Dehoux et al., 2004). Alternative studies have demonstrated that the level of these ubiquitin ligases is increased during rapid muscle development due to increased protein breakdown observed during catabolic conditions (Lang et al., 2007). In the present experiment, the rapidly-growing coho salmon appeared to be breaking down and turning over muscle more extensively during growth, thereby showing an increased level of only certain degradation factors and ubiquitin ligases. Conversely, the expression of these genes was basically identical between wild-type and Tran-RR fish, demonstrating that reduced capacity of growth hormone effects is regulated by both genetics and available energy.

4.2. Liver

There were a number of gene expression changes in the liver that seem to be tied specifically to the transgenic modification of the fish. Specifically, when the expression of immunological factors in the liver was examined, we found that *CXCL-8* was significantly up-regulated in transgenic fish, and was expressed lowest in domesticated fish. In a similar manner, *TNFα* was expressed lowest in domesticated fish, while inversely *NFκβ* was expressed highest in non-transgenic fish. Prior research has suggested that growth hormone-transgenic fish might have a slightly depressed immune system (Jhingan et al., 2003), yet here our findings imply that certain subsystems of the innate immune system actually might be up-regulated. Similar studies in growth hormone-transgenic common carp suggested that enhanced growth hormone production stimulated non-specific immune functions (Wang et al., 2006). However, steady-state increased immune expression may not predict how well the animals will respond upon infection with certain pathogens. However, previous analysis of the expression of these genes in rainbow trout did find a correlation between expression and resistance to bacterial and viral pathogens (Overturf and LaPatra, 2006). Glutathione peroxidase expression is another indicator of cellular health in animals (Michiels et al., 1994). Here again, it appears that increased growth rate, likely correlated with enhanced growth hormone levels, is involved with glutathione peroxidase production (Leggatt et al., 2007). In GH-knockout mice, glutathione peroxidase production has been linked with muscle satellite cell proliferation and increased life span (Muller et al., 2007).

Therefore, up-regulation of growth hormone production might be beneficial to cells of transgenic and growth-selected salmonids.

The expression of certain growth regulatory factors in the liver was similar to results of earlier studies with these fish regarding IGF-I and II (Devlin et al., 2009a,b). *IGF-I* expression levels were slightly but significantly increased in Tran-RR fish compared to wild-type. This rise could be through potential energy enhancement from the growth hormone transgenesis providing increased utilization of dietary energy (Oakes et al., 2007; Higgs et al., 2009). Domesticated fish demonstrated increased levels of *fox01* and *IGF-II* in the liver, which could mediate some of the regulatory metabolic differences that were found between selected and transgenic fish.

Similar differences to those described above were noticed upon evaluating several energy metabolism-specific genes in the liver. Tran-FF and domesticated coho salmon showed significantly increased levels of expression for *pyruvate dehydrogenase*, *aspartate aminotransferase*, and *PPARγ* genes. These genes all are involved with metabolic regulation, fatty acid regulation, and amino acid metabolism (Kersten et al., 2001; Wu, 1998). The enhanced expression of these genes most likely is linked to the increased muscle turnover for these faster-growing animals, as was suggested from the expression patterns of the growth and muscle regulatory genes evaluated in the muscle of these fish. In mammals, mTOR (mammalian target of rapamycin) is a key system involved with integrating the input from multiple upstream pathways, including growth hormones, cellular nutrient and energy levels, to regulate transcription, cellular growth, proliferation, and protein synthesis (Tokunaga et al., 2004; Corradetti and Guan, 2006). REDD-1 is a primary inhibitor of mTOR and could be functioning to reduce TOR activity in fish. Prior studies have found that stimulatory signals, such as high nutrient levels, reduce TOR kinase activity (Kim et al., 2002). This finding fits with the high levels of expression seen for *REDD-1* in Tran-FF fish, but is difficult to reconcile with TOR being expressed at a reduced level in domesticated compared to wild-type and Tran-RR fish. This result may reflect differences in regulation of energy and management of growth between selected domesticated fish and growth hormone-transgenic coho salmon.

5. Conclusion

From these studies, it appears that domestication and enhancement of unselected stocks through growth hormone transgenesis improve growth mainly through the modulation of similar related gene pathways to achieve analogous physiological changes in the animal. Restricting the diet and thereby decreasing the available energy for the fish appears to severely depress metabolism and growth-related mechanisms of the fish, such that their growth potential becomes similar to that of wild-type coho salmon which possess reduced genetic potential to respond to an increase in available energy. The findings presented here show similar regulation in muscle development and regulation between domesticated and growth hormone-enhanced fish fed to satiation. These findings suggest that targeted physiological differences such as changes in cellular immunity or muscle development may be possible with markers linked to the activity of specific genes. Further research utilizing stocks of different genetic backgrounds potentially could identify genes that enhance growth more efficiently.

Notable differences between domesticated and Tran-FF fish appeared in the expression of the muscle differentiation factors *cee* and *Pax7*, certain innate immunological genes in the liver, expression of certain energy metabolism-related genes, but probably most distinctly with REDD-1. Prior studies have found that there is a greater number of transcriptional changes overall in transgenic fish compared to domesticated fish and suggest that these changes arise from dysregulation of pathways due to fish having to cope with this dramatic metabolic change in a single generation.

Acknowledgements

The authors thank USDA-ARS biological technician Karen Frank at the Hagerman Fish Culture Experiment Station in Hagerman, Idaho for her assistance in sample analysis and the fish culture staff at the Transgenic Fish Facility at Fisheries and Oceans Canada's Centre for Aquaculture and Environmental Research (CAER).

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